



Innovations and Challenges in Foot-and-Mouth Disease Vaccine Development: Bridging Traditional Methods with Emerging Technologies

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Abstract | Foot-and-mouth disease (FMD) is one of the most economically impactful emerging viral disease infecting cloven-hoofed animals. Vaccination is critical to controlling FMD infection and reducing its transmission. FMD vaccinations have been able to control and eradicate the infection in various countries or regions, however, it is still endemic in some areas of Asia and Africa. FMD vaccine technologies have substantially developed during past decades, yet more robust and flexible technologies suitable for developing countries' situations are needed. This review offers an in-depth analysis of the status of FMD vaccine development, highlighting both conventional and innovative technologies that could enhance vaccination effectiveness and availability. Various types of FMD vaccines have been discussed, including inactivated, attenuated, virus-like particles, peptide, and nucleotide-based vaccines, highlighting their properties, drawbacks, and recent advancements. Besides, the use of adjuvants and delivery systems to boost vaccine performance was also reviewed. Furthermore, we explore state-of-the-art methodologies such as exosome-based vaccines, cell-free protein synthesis systems, self-amplifying RNA vaccines, and the application of artificial intelligence (AI) and computational biology in vaccine design. These methodologies may offer solutions for the challenges posed by the foot-and-mouth disease virus's high mutation rate, antigenic variability, and the need for enhanced protection and thermostability.

Keywords | Foot-and-mouth disease, Vaccine, Exosomes, Cell-free, Artificial intelligence, Adjuvant

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INTRODUCTION

Emerging viral strains have produced both past and present outbreaks, posing a substantial threat to an-

imal health and productivity, particularly in developing countries, where economic losses are a major concern. One way to control viral infections is through vaccinations. Vaccinations are mainly used to enhance the immune response

against viral infections and reduce transmission between the hosts (Aida *et al.*, 2021; Pollard and Bijker, 2021; Z. Zhang *et al.*, 2022). However, some viruses tend to have continuous variations leading to the demand for continuous development of vaccines.

One of the most important viral infections in livestock is foot-and-mouth disease (FMD), an emerging viral disease that affects cloven-hoofed wildlife and domestic animals including cattle, buffalo, goats, sheep, and pigs (Jamal and Belsham, 2013). FMD is caused by foot-and-mouth disease virus (FMDV), a member of *Aphthovirus* genus in the *Picornaviridae* family. FMDV encloses seven distinct serotypes named A, O, C, Asia-1, and South African Territories (SAT) 1-3 (Grubman and Baxt, 2004). The rapid mutation rate of the virus, along with the presence of numerous serotypes and subtypes, leads to continuous antigenic drift, that complicates vaccine efficacy and development. Even after recovering from an infection caused by one serotype, animals may lack immunity against others, imposing continuous vaccine innovation (Mahapatra *et al.*, 2015).

Serotype O, the most frequent FMDV strain, caused 70% of global outbreaks (Samuel and Knowles, 2001), and this case remained mostly unchanged (Belsham, 2020; Brito *et al.*, 2017). As stated, serotypes vary greatly, especially among viruses from different regions. Except for Asia-1, nucleotide sequence analysis has divided viral serotypes into distinct topotypes with distinct geographic distributions (KNOWLES *et al.*, 2016). Although Asia-1 FMDV has reached Greece and SAT-2 FMDV has entered Egypt, the SAT and Asia-1 serotypes rarely migrate outside their normal locations (Brito *et al.*, 2017). O and A serotypes are common in several regions (Blacksell *et al.*, 2019; Knowles *et al.*, 2005). The wide geographical distribution pushed the necessity of the development and production of efficient vaccines throughout the years in intent to control the FMDV in conjunction with other control measures.

FMD vaccine development started with inactivated virus formulations, in the early 20th century. This development gave hope for control of this economically important disease. Inactivated vaccines faced multiple challenges such as the requirement of multiple dosages and lack of cross-serotype protection. Those challenges change the focus to develop a different type of vaccine, live attenuated vaccines, that offer a stronger immune response and long-lasting protection. Despite their advantages over inactivated vaccines, they posed the risk of reverting to virulence. Late 20th century and early 21st century, a shift in the paradigm of FMD vaccine development has become noticeable through the development of virus-like particles, peptide-based vaccines, and nucleic acid vaccines. Since then, those vaccines have been in continuous development till now however there are a very limited field data on their efficacy (Lu *et al.*, 2022).

Even though vaccination policies eradicated FMD from North America, Western Europe, and parts of Asia (Paton *et al.*, 2021), the disease remains endemic in Asia and Africa. Areas of endemic FMD are facing multiple challenges that hinder disease eradication. The main obstacles resemble the high cost of vaccines, logistical problems related to vaccine distribution, insufficient vaccination coverage, inadequate epidemiological understanding of the disease, and socio-economic factors affecting vaccination strategies (Mashinagu *et al.*, 2024; Sinkala *et al.*, 2014; Sultanov *et al.*, 2023).

Understanding the molecular structure and genetics of FMDV is crucial for developing effective vaccines. The FMDV genome consists of a single-stranded positive-sense RNA of approximately 8,500 nucleotides, which encodes for four structural proteins (VP1, VP2, VP3, and VP4) and eleven non-structural proteins (Figure 1) (Grubman and Baxt, 2004; C. Li *et al.*, 2018). The structural proteins form the viral capsid, which is essential for protecting the viral RNA and facilitating entry into host cells (Belsham, 2020). The presence of specific genetic features, such as the internal ribosome entry site (IRES) in the 5' untranslated region (UTR), is critical for viral replication and protein synthesis (Sarry *et al.*, 2022). Recent research has highlighted the importance of these molecular details in guiding vaccine design, as they can influence the immunogenicity and stability of vaccine candidates (Lu *et al.*, 2022). For instance, VP1 is a major antigenic determinant and is critical for inducing neutralizing antibodies. Targeting these proteins in vaccine formulations can enhance the immunogenicity of the vaccines (Kim *et al.*, 2013). As well, studying the genetic makeup of different FMDV strains, researchers can design vaccines that include multiple serotypes or develop universal vaccines that can provide broader protection (Hwang *et al.*, 2023; Shin *et al.*, 2024). Furthermore, Understanding the molecular structure allows for the identification of stabilizing agents or modifications that can enhance the stability of vaccine formulations, ensuring they remain effective during storage and administration (Jin *et al.*, 2024). Moreover, Knowledge of FMDV's molecular structure allows researchers to select or design adjuvants that work synergistically with the vaccine components, improving overall vaccine efficacy (Z. Liu *et al.*, 2019). Knowledge of the viral genome and its potential for reversion to virulence (especially in live-attenuated vaccines) allows for the design of safer vaccine candidates (Medina *et al.*, 2023).

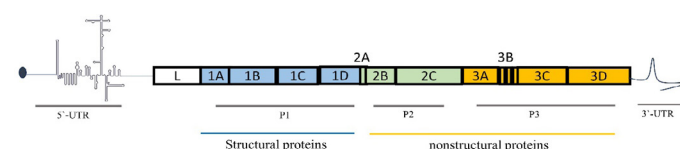


Figure 1: FMDV genomic structure showing the 5' UTR, P1, P2, P3 and 3' UTR segments.

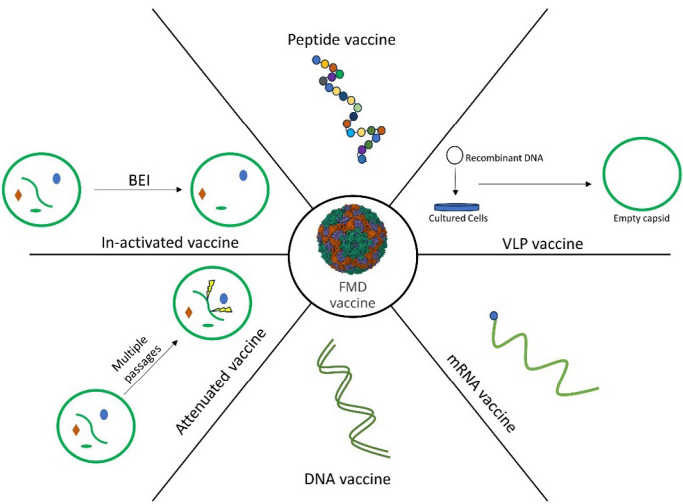


Figure 2: Types of FMDV vaccines.

Herein we present, the past and current vaccine studies of one of the most important viral infections in livestock caused foot-and-mouth disease (FMDV). The review will also highlight new vaccine technologies with the potential for FMD vaccine development.

FMDV CURRENT VACCINE RESEARCH

Historically, vaccine development for FMD has evolved significantly. The first vaccines were developed in the early 20th century, primarily using inactivated virus. Over the decades, various vaccine types have emerged, including live-attenuated, virus-like particles (VLPs), peptide-based, and nucleic acid vaccines (Figure 2). Each vaccine type presents unique advantages and challenges (Table 1), particularly in terms of safety, efficacy, and production costs. For instance, while inactivated vaccines are widely used, they often require multiple doses and do not provide long-lasting immunity. Conversely, live-attenuated vaccines may offer better immune responses but carry the risk of reversion to virulence.

Vaccine development for FMDV has attracted wide interest during the last twenty years. Research has focused on addressing the issue of universal vaccine coverage. During vaccine development, certain criteria should be taken into consideration such as safety, long immunity from a single dosage with lasting effect, cheapness, and compatibility with the DIVA principle. Next, we will discuss the different types of commercial and in-development vaccines for FMDV.

Table 1: FMDV developed vaccine advantages and limitations.

Vaccine Type Advantages		Limitations
Inactivated	Currently the only commercially available vaccine Proven efficacy in controlling FMD outbreaks	Require multiple doses for long-lasting immunity Need for cold chain storage and transport Potential for non-protective antibody formation if vaccine integrity is compromised Require biosafety level III facilities for production to prevent accidental release of FMDV Lack of cross-protection between serotypes and even strains within the same serotype necessitates the inclusion of multiple virus isolates, which can stress the animal immune system
Live-Attenuated	Higher stability compared to inactivated vaccines Lower risk of reverting to virulence with certain attenuation methods (e.g., gene deletion)	Potential for reversion to virulence, especially with codon deoptimization methods where recombination events can occur between attenuated and wild-type strains Careful attenuation is required to achieve the right balance between safety and immunogenicity
Virus-Like Particles (VLPs)	Resemble natural virus conformation, inducing neutralizing antibodies Safer than live viruses as they lack virulence genes and infectious agents Can induce both T-cell and B-cell responses	Require multiple administrations to induce effective immune responses Stability issues with some VLP constructs, necessitating modifications to increase hydrophobicity
Peptide-Based vaccines	Safe as they do not contain any virulence genes or infectious agents Do not require cold chain storage and transport	Limited immunogenicity, often requiring carrier proteins and multiple antigenic variants to enhance immune responses Prone to enzymatic digestion
RNA Vaccines	Compatible with rapid vaccine development for viral pandemics and epidemics Safer than DNA vaccines as they do not involve genome integration Can induce both cell-mediated and humoral immunity	Delivery challenges to ensure sufficient uptake of RNA into host cells Potential for excessive immune responses and stability issues

FMDV inactivated vaccine is considered the only available commercial vaccine nowadays. Various methods have been used for FMDV inactivation, in the beginning, formalin was used to inactivate the living virus (LEFORBAN and GERBIER, 2002). Today, FMDV inactivation is performed by treating the virus with Binary ethylenimine (BEI) (Kamel *et al.*, 2019; Parida, 2009). Other methods were also used to inactivate the virus such as N-acetyl ethyleneimine, formaldehyde (Barteling and Woortmeyer, 1984), virion-associated endonuclease (Diaz-San Segundo *et al.*, 2016), or non-chemical hydrostatic pressure (Ishimaru *et al.*, 2004). The inactivated FMD Vaccine is very effective as it induces strong immunity and is safe as it poses no risk of infection to vaccinated animals.

The inactivated vaccine can be produced in large quantities in BHK-21, a continuous cell line for FMDV production. BHK-21 is cultured in high density and can produce a high virus titer (Lu *et al.*, 2022). Several approaches have been taken to produce higher FMDV titer while keeping the lowest possible cell density through changing media formation (Park *et al.*, 2021), alternating cell genetic material by expressing receptors that increase the match between virus and cell (Harvey *et al.*, 2022), or inhibiting genes responsible for decreased virus replication (Hou *et al.*, 2022).

One of the main disadvantages of inactivated vaccines is the need for biosafety III facilities to avoid FMDV release during vaccine production (Kamel *et al.*, 2019). The inactivated vaccine also requires multiple doses to achieve long-lasting immunity as compensation for the degradation of intact viral particles before vaccine administration (Kumru *et al.*, 2014). The degradation of intact capsid leads to the formation of pentamers that show very poor immunity and induce the formation of non-protective antibodies (Doel and Chong, 1982). Some serotypes, such as O and SAT2, virus particles are unstable. Therefore, the stabilization of the vaccine is necessary for an effective vaccine and longer storage period. Additives such as metal ions (L. Zhang *et al.*, 2022), peptides (Volkin *et al.*, 1997), polysaccharides (Hwang *et al.*, 2020), or amino acid substitution (Biswal *et al.*, 2022) have been used to improve FMDV stability.

Different virus isolates could be included in the vaccine due to the lack of cross-protection between different serotypes or even between strains within the same serotype such as circulating isolates in Egypt (Bazid *et al.*, 2023), East Africa (Childs *et al.*, 2023), and China (Z. Zhang *et al.*, 2023). Therefore, the vaccine can be monovalent or multivalent based on the circulating strain where the vaccine will be used (Parida, 2009). Including such a high number of isolates in the vaccine can introduce stress to the animal immune system.

Although inactivated vaccine wide usage, its disadvantages outweigh its strengths. The incompetence of this vaccine also showed low performance in field settings, where they failed to prevent viral replication in vaccinated animals, the formation of virus carrier hosts, and the continuation of FMDV spread (Belsham, 2020).

LIVE-ATTENUATED VACCINE

FMDV vaccines can be produced through attenuation either by a conventional method such as passing into multiple cultured cells or through new methods such as gene manipulation or deoptimization (Kamel *et al.*, 2019). The attenuated vaccines have higher stability and cost-effectiveness than inactivated vaccines. Attenuated vaccines induce strong and long-lasting immune responses using fewer doses than inactivated vaccines (Singh *et al.*, 2019).

Investigation of virulent viral genes and identification of their inactivation is an important step for developing a live attenuated vaccine. Frame-shift mutation or gene deletion showed no clinical signs or viremia in vaccinated animals. An example of those virulent genes is the leader protease gene (Brown *et al.*, 1996). Gene manipulation attenuation attempts focused on the generation of viruses without a leader protease gene. This method proved to produce a strong protective antibody response in mice and swine (Chinsangaram *et al.*, 1998; MASON *et al.*, 1997). Another strategy for attenuation is codon deoptimization (Pereira-Gómez *et al.*, 2021). Codon deoptimization of the P1 coding sequence was used for different serotypes of FMDV (Diaz-San Segundo *et al.*, 2016; Medina *et al.*, 2023). Some issues were reported in this attenuation strategy. For instance, the deoptimization process must be carefully tuned to gain the required attenuation without affecting protective immune response induction (Medina *et al.*, 2023). Another concern is regarding the reversion of virus virulence. Some recombination events were reported between codon-deoptimized virus and wild-type strains within the host body (Spinard *et al.*, 2023). That recombination led to the reversion of codon deoptimized vaccine to wild-type form.

Attenuated vaccines provide great potential for control of FMD; however, their attenuation techniques may face some challenges. The conventional method is time-consuming and the resulting vaccine may not provide the required immune response due to random variation of viral genome. The new methods are very complex and require a high technicality, in addition to the chance of recombination and risk of reversion to virulence. Besides the limitations of attenuation strategies, the production process needs careful monitoring to prevent any potential outbreaks from vaccine strains (Singh *et al.*, 2019).

VLPs are empty viral particles composed of virus structural protein and do not include virus nucleic acid. The VLP structure resembles the natural virus conformation that induces the production of neutralizing antibodies with extra safety of no viral replication or presence of virulence genes (Li *et al.*, 2023; Quattrocchi *et al.*, 2020; Song *et al.*, 2024). The VLP vaccines can also contain multiple antigens, enabling them to target various FMDV serotypes, which increases their effectiveness (Rangel *et al.*, 2021).

Two approaches are followed when introducing VLPs as a vaccine; 1) delivering capsid genes to host cells through a viral vector, 2) producing empty capsid in culture media (bacterial, yeast, plant, or mammalian cells), and then administering as a vaccine. Researchers developed different VLPs using various platforms (Kushnir *et al.*, 2012).

Viral-based vaccine was the first platform used for VLPs production. Adenovirus vector-based vaccines have been developed to produce FMD VLP vaccine that was able to stimulate specific immune responses. This method depends on replacing E1A/E1B adenovirus genes with the structural genes of FMDV and 3c protease important for capsid formation (Mayr *et al.*, 1999). This method was applied to different FMDV isolates including A24/Cruzeiro/BRA/55 (Moraes *et al.*, 2002), O/Manisa/TUR/69 (Fernandez-Sainz *et al.*, 2017), and O1/ Campos/Brazil/58 (Ziraldo *et al.*, 2020).

Phage display vaccine is another example of a viral-based vaccine. It depends on displaying FMDV VP1 on a phage capsid to induce the production of specific antibodies in mice (Wong *et al.*, 2022). Another method, a DNA-based vaccine, uses a plasmid vector to harbor the DNA sequence of FMDV structural genes under the control of specific proteins. DNA vaccines have many advantages over other types of VLP production platforms (Lu *et al.*, 2022). They are safe as they do not contain any virulence genes or infectious agents and they do not require a specific cold chain for storage and transportation. Moreover, they can induce T and B-cell immune responses while keeping the immune system unstressed. However, DNA vaccines require multiple administrations to induce an effective immune response (Dory *et al.*, 2009). Co-injection of the plasmid containing VLP genes with plasmid encodes granulocyte-macrophage colony-stimulating factor (GM-CSF) could aid in the stimulation of a stronger immune response (Fowler *et al.*, 2012).

E. coli and mammalian expression systems have been used to construct the VLP outside the host cells. In this approach, the FMDV capsid genes are amplified, tagged with a small ubiquitin-like modifier (SUMO), and inserted into a proper expression vector to be expressed in *E. coli* (Xiao

et al., 2021). The expressed proteins are then purified and used to form the VLP vaccine. Modifications of amino acid sequences were introduced to increase the stability of virus particles through the elevation of VLP hydrophobicity (L. Li *et al.*, 2021). Other bacterial species such as attenuated *Salmonella typhimurium* (Zhi *et al.*, 2021) and *Lactococcus lactis* (Liu *et al.*, 2020) were used for oral vaccine administrations and expression of VLP within host cells. In mammalian systems, transient gene expression was used to produce VLP for A2001 Argentina and O/man strains. This platform is cheap, allows repeatability, production of multiple vaccine copies, and rapid production of protein numbers (Puckette *et al.*, 2022).

VLP-based vaccines are one of the next-generation vaccines that show a great promise to improve FMD control, however, there is limited data on its efficacy in the field premises. This lack of data affects its acceptance and usage. It is also very costly and complex to produce and requires advanced biotechnology methods that affect its large-scale production (Choudhury *et al.*, 2021).

PEPTIDE-BASED VACCINES

Peptide vaccines are small segments or subunits of the viral protein sequence that can induce T-cell or B-cell reactions. They can be produced at a low cost and safe manner as they do not require the propagation of live viruses. Peptide-based vaccines can be tailored to produce immune responses against specific epitopes (Forner *et al.*, 2021). Subunit peptide vaccines such as FMDV VP1 C-terminus (200-213) or G-H loop (141-160) were only able to induce limited T-cell responses (Mansuroğlu *et al.*, 2020). T-cell epitope has been identified in VP4 residues (20-34) that have been used in peptide vaccines for T-cell stimulation (Blanco *et al.*, 2000). It was proven that a mixture of antigenic variant peptides was more immunogenic than using a single peptide. Therefore, Multiple antigen peptide systems (MAP), dendrimers that are composed of one FMDV T-cell epitope branched to four sets of B-epitopes (Lu *et al.*, 2022), showed more immunogenicity than linear peptides which are prone to enzymatic digestion (Lu *et al.*, 2022). Limited immunogenicity of linear peptides can also be enhanced using adjuvant. The peptide sequence is linked to carrier proteins such as bacterial toxoid or ovalbumin. These carrier proteins should be safe, and potent, can be produced on a large scale at a low cost, and have immunogenic properties (Kamel *et al.*, 2019).

RNA VACCINES

mRNA vaccines are used through direct transfection of viral mRNA to host cells to produce viral protein. The produced protein induces cell-mediated and humoral immunity based on the contained epitopes (Cagigi and Loré, 2021; Fazel *et al.*, 2024). mRNA vaccines are very compatible with viral pandemics and epidemics (Fazel *et al.*, 2024).

In contrast to DNA vaccines, RNA vaccines do not impose host genome integration (Wang *et al.*, 2004). It is also safe as it does not involve propagation or virus replication (Par-di *et al.*, 2020). FMDV mRNA vaccine was reported for immunization in swine. dsRNA of FMDV 3' non-coding region was able to induce INF- α and β activation in the SK-6 cell line, and natural immune response in newborn mice (Pulido *et al.*, 2010; Rodríguez-Pulido *et al.*, 2011). Non-infectious RNA consisting of IRES elements was used as an adjuvant for the conventional FMDV vaccine. This method was proved to enhance the immune response in mice and pigs, besides the enhancement of FMDV-specific antibody titer (Borrego *et al.*, 2017). The effective delivery of mRNA vaccine into host cells is still a challenging task and requires a sophisticated delivery system. Furthermore, booster doses are required as mRNA vaccines do not generate long-lasting immune responses compared to traditional methods (Al Fayed *et al.*, 2023). Additionally, mRNA vaccines require cold chain system, as it is prone for degradation. The purity of the mRNA vaccine is also a major consideration, the presence of dsRNA can result in the inhibition of the protein translation and degradation of mRNA strand (Jamous and Alhomoud, 2023).

ADJUVANTS AND MODE OF DELIVERY

FMDV VACCINES ADJUVANTS: The use of effective adjuvants, a suitable delivery system, and an administration route enable a successful vaccine development. Adjuvants are various substances that aid in the enhancement of the host's adaptive immunity against the vaccine, through the activation of innate immune cells (Pulendran *et al.*, 2021). They activate antigen-presenting cells (APC) through antigen-presenting and co-stimulatory signals (Coffman *et al.*, 2010). Adjuvants can be varied from small molecules to complex natural or chemically synthesized particulate molecules that are called immunostimulants (McKee *et al.*, 2007). Immunostimulants interact with pattern recognition receptors (PRRs) presented on APCs activating their maturation. Mature APCs then present the antigens and upregulate the expression of co-stimulatory and cytokine signals leading to the initiation of adaptive immune response. Each immunostimulant will interact with different PRRs and induce different cytokine pathways. The detailed mechanism for the different types of adjuvants and delivery systems has been extensively discussed in this review (Zhao *et al.*, 2023). Following, we will discuss the commonly used adjuvants with developed vaccines against FMDV.

OIL ADJUVANTS: Oil-based adjuvants are known to elicit a strong immune response due to their dual function as antigen delivery and immune system stimulatory. Different types of adjuvants were used in conjugation with FMDV inactivated vaccine. MF59, MONTANIDE ISA-206, and ISA70 are notable oil-in-water emulsion adjuvants. MF59

demonstrated the ability to induce Th2-biased immune response and weak induction of Th1 responses (A *et al.*, 2023; O'Hagan *et al.*, 2021). The usage of ISA70 with inactivated FMD vaccine produced acceptable immunogenicity, however, the titer of the antibody produced by MF59 was higher (A *et al.*, 2023). MONTANIDE ISA-206, widely used in veterinary viral vaccines, was observed to have a degradable effect on FMDV particles extracted from vaccines into smaller particles (Harmsen *et al.*, 2015). Despite their efficacy, oil adjuvant has serious side effects including necrosis, swelling, and hemolysis at the site of injection (Lu *et al.*, 2022).

ALUMINUM HYDROXIDE ADJUVANT: Aluminum hydroxide adjuvants work on the production of the immune response that leads to the release of IgE and IgG antibodies (Marrack *et al.*, 2009). Aluminum-based adjuvants are safe and reliable; however, they have multiple drawbacks resembling as causing erythema and allergic reactions. Like oil adjuvants, Aluminum hydroxide functions as both a delivery system and an immunostimulant. It enhances the bioavailability of the vaccine through the sustainable release of its antigens. Therefore, it increases the antigen presentation of vaccine proteins. In addition, it interacts with the immune cells by activating specific PRRs resulting in cytokine release and Th2 immune response (HogenEsch *et al.*, 2018; Sokolovska *et al.*, 2007). Despite the benefits, the induced immune response by these adjuvants is not strong enough. This limitation can be managed by improving the adjuvant formulation through the inclusion of saponin or the preparation of nano aluminum-based adjuvants (Moyer *et al.*, 2020; Peng *et al.*, 2020). Aluminum hydroxide gel combined with saponin and oil adjuvant showed a better immune response than a mixture without oil adjuvant when added to the trivalent FMD vaccine (Ayele *et al.*, 2023).

TOLL-LIKE RECEPTOR (TLR) AGONISTS: TLR agonists are potent immunostimulants that interact with TLRs, help in antigen presentation, increase the expression of co-stimulatory and cytokine signals, and eventually enhance adaptive immune response (Lind *et al.*, 2022; Ong *et al.*, 2021). Cell surface TLRs such as TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6 can recognize pathogen membrane components. The interaction of microbial lipids, lipoproteins, and surface proteins with these TLRs promotes the production of pro-inflammatory cytokines and induction of the Th1 or Th2 immune response (Luchner *et al.*, 2021). Intracellular TLRs including TLR3, TLR7, TLR8, and TLR9 interact with nucleic acids, leading to the production of interferon that may induce Th1 cell differentiation and enhance antigen presentation by dendritic cell (DC) (Aleynick *et al.*, 2019; Desmet and Ishii, 2012).

Several TLR agonists were used as adjuvants with the FMDV vaccines (Aleynick *et al.*, 2019). For instance, CVC1302, a mixture of muramyl dipeptide (MDP), monophosphoryl lipid A (MPL), and B-glucan was able to induce humoral immunity when combined with FMD multi-epitope vaccine, though it was insufficient to activate cellular immunity against FMDV. TLR4 agonists such as dimethyldioctadecylammonium bromide (DDA) liposomes were reported to coat VLPs effectively and enhance Th1 response (Du *et al.*, 2021; Kim *et al.*, 2020). Additionally, heparin-binding hemagglutinin (HBHA) from *Mycobacterium tuberculosis*, another TLR4 agonist, has been utilized for increasing the stability and solubility of multiepitope vaccines and elevate the expression of IL-4, IL-6, IL10 and IL12p70 cytokines (Lei *et al.*, 2020). Polysaccharide extracted from *Cistanche deserticola* (CPCD), combined with inactivated FMD vaccine, was employed to activate DC by interacting with TLR-2 and TLR-4 and triggering MAPK and NF- κ B pathways (Q. Li *et al.*, 2021).

NON-CODING RNA (ncRNA): ncRNA is a sequence of RNA that can form a secondary structure like that of the non-coding sequence of FMDV genes and can interact with the intracellular TLRs including TLR3. This interaction facilitates the induction of proinflammatory cytokines and interferons, thereby promoting a strong Th1 immune response. Also, when the IRES sequence is used with the multiepitope vaccine, can cause an increase in the amount of INF- γ producing cells. However, in return, the specific humoral response is reduced. To optimize the effectiveness of the ncRNA, a suitable delivery system should be used to protect the RNA from degradation (Cañas-Arranz *et al.*, 2020; Rodríguez-Pulido *et al.*, 2022).

DELIVERY SYSTEMS

INORGANIC NANOMATERIALS: Gold nanoparticles (AuNPs) unique physicochemical properties including size, morphology, hydrophobicity, charge, composition, and homogeneity give them the edge to be the most common inorganic nanomaterials used as a delivery system. Their surface characteristics can be tuned through chemical modification and materials used during the synthesis process, allowing for a targeted immune response (Dykman, 2020; Li *et al.*, 2018). AuNPs have been employed to be used as a delivery system for FMDV VP1 with particles ranging from 8-17 nm in diameter. Such a delivery system was able to provoke a high level of antibody production (Chen *et al.*, 2010). Gold nanocages (AuNCs) were also used to deliver and work as an adjuvant for the FMD VLP vaccine. The formulated vaccine was administrated intramuscularly to mice. The VLP-AuNCs were able to stimulate DCs by interacting with TLR4 and enhanced T-cell proliferation significantly (Teng *et al.*, 2021).

Similarly, mesoporous silica nanoparticles (MSNs) have been employed as a delivery system for FMDV vaccines. MSNs were synthesized using iron oxide to form extra-large mesopores. Surface modification of MSNs using polyethyleneimine (PEI) enabled efficient loading of proteins of various sizes leading to the enhancement of antigen presentation (Nguyen *et al.*, 2019). Dendritic MSNs were utilized to be loaded with B2T MAP peptide, showing high and sustained immune response due to the prolonged peptide release (An *et al.*, 2021). FMD VLP vaccine was also loaded to MSNs and showed a greater and higher immune response compared to VLP alone after 7 days of immunization. The sustained release of vaccine caused by MSNs helped reduce the amount of injected vaccine (Bai *et al.*, 2019). FMDV recombinant plasmid was loaded on amino-functionalized MSNs for induction of mucosal immunity through intranasal delivery. Unfortunately, the conjugate was not effective in stimulating mucosal and systemic immune responses. This was reasoned for the large size of plasmid DNA to be incorporated into MSN particles, the low adhesion of particles to mucous membrane, particle size, or amount of dose administrated (Zheng *et al.*, 2020).

POLYMER PARTICLES: Polymer particles, either natural or synthetic polymers, were also widely utilized as a delivery system and played a critical role in the sustained release of its loaded antigens (Zhao *et al.*, 2023).

Chitosan, a natural polymer, is extensively used as a vaccine delivery due to its bio-adhesive properties and cationic charge. This bio-adhesive nature allows it to adhere to the mucosal surfaces and activate continuous immune cell simulation. They are also capable of delivering nucleic acid vaccines by forming electrostatic interactions with them (Iqbal *et al.*, 2003; Wu *et al.*, 2020). Zinc-cleated chitosan particles have been utilized to adsorb the inactivated FMDV vaccine onto their surfaces, increasing the vaccine's thermal stability, and promoting cellular and humoral immune responses (Li *et al.*, 2020).

Synthetic polymer particles, such as poly(lactic-co-glycolic acid) (PLGA), offer higher reproducibility and more precise control over antigen release. PLGA can be used to encapsulate the vaccine material and increase antigen presentation by dendritic cells (Kim *et al.*, 2018). Coating PLGA with chitosan loaded with FMDV recombinant DNA plasmid along with CpG oligonucleotide as an adjuvant has been shown to improve immune response in mucosal cells (Yang *et al.*, 2021). Mannose-modified chitosan-coated PLGA loaded with an expression plasmid containing FMDV T and B-cell epitopes was used as a nasal vaccine. The vaccine was able to induce mucosal and systemic immune responses in mice (Li *et al.*, 2024).

CAGED PROTEIN NANOPARTICLES: Caged protein nanoparticles consist of repeated structural motifs that are assembled in a series with antigenic epitopes incorporated into their structure. They showed the ability to traffic antigens to the lymph nodes and activate B-cells when linked with their receptors (Neek *et al.*, 2019). For instance, Ferritin nanoparticles, have been employed to enhance FMDV antigens stability as well as promote the proliferation of memory and effector T cells causing a more robust immune response (Chen *et al.*, 2020). Another ferritin nanoparticle was used for displaying of the VP1 neutralizing epitope (aa 140-158) on the surface of the nanoparticle. The method was able to elicit partial protection in mice and guinea pigs that were consistent with immunity induced by the FMDV-inactivated vaccine (Lu *et al.*, 2024).

Lumazine synthase (LS) and *Quasibacillus thermotolerans* encapsulating (QtEnc) are other examples of caged protein nanoparticles. They have been incorporated with recombinant VP1 from serotypes A and O to generate multimeric nano vaccines. LS-VP1 and QtEnc-VP1 nano vaccines were able to induce a high level of IgG antibody, which was 100 times more than produced by a single antigen. The immune response to those vaccines was more Th1-biased response and production of neutralizing antibodies (Peng *et al.*, 2024).

FRONTIERS OF NOVEL VACCINE TECHNOLOGIES

Over the past decades, research on FMD vaccines has undergone constant improvement. The primary focus of the researcher's studies was to enhance the effectiveness of the vaccination and address the variety of virus serotypes. Nevertheless, various constraints have been outlined regarding the vaccine development endeavors, as described in previous sections. In recent years, there has been significant progress in the development of new technologies. These technologies were created to enhance biological and therapeutic applications. These tools have the potential to bring new opportunities for advancements in several sectors of the FMDV vaccination industry. In the subsequent section, we will explore these technologies that have been utilized or have potential uses in the field of vaccine research and development (Table 2).

EXOSOMES FOR VACCINE DEVELOPMENT: Extracellular vehicles (EVs) are small lipid vesicles of different sizes released by almost all types of host cells. They were identified as key players involved in intercellular signaling (Hendrix *et al.*, 2023; El Safadi *et al.*, 2024). EVs are categorized into three subtypes based on size and function: 1) EVs with sizes around 5 μm are blebs and apoptotic bodies; 2) EVs with sizes between 100 and 1000 nm are ectosomes or microvesicles; 3) exosomes are between 30

and 150 nm diameters (Doyle and Wang, 2019; Kalluri and LeBleu, 2020).

Interest has been increased towards exosomes because of their ability to act as natural infection pathways and specifically target immune system cells (Besse *et al.*, 2016). These vesicles naturally encapsulate a variety of cellular molecules such as lipids, RNA, DNA, MHCs, enzymes, heat shock proteins, and transcription factors (Hao *et al.*, 2021).

Exosomes demonstrated significant capabilities in advancing biomedical applications, particularly in the field of vaccines and therapeutics. Dendritic cell-derived exosomes, which contain a large amount of MHCI and MHCII molecules, can activate mature DC to prime T-cells (Lindenbergh *et al.*, 2019). Exosomes may also act as antigen-presenting platforms, delivering MHC complexes or antigens to other cells (Lindenbergh *et al.*, 2020). Moreover, exosomes have shown promise as nano-adjuvant. In a study, exosomes extracted from in vitro stimulated monocytes by lipopolysaccharide (LPS) endotoxin showed non-specific immunostimulation. But, when combined with hepatitis B virus (HBV) recombinant antigen, it boosted the immune response through raising the level of INF- γ (Jesus *et al.*, 2018). Exosomes were also employed as a delivery method for nucleic acid due to their nano-carrier characteristics (Naseri *et al.*, 2018). However, natural exosomes face some drawbacks including the inability to target specific tissues or cells, low solubility, and short half-life (Malekian *et al.*, 2023). Therefore, modification of exosomes is necessary to avoid inconsistent results.

There are two ways for exosome modification, either through parental cell-based or post-isolation modification (Jafari *et al.*, 2020). Those methods have been used to load required antigenic proteins and peptides onto exosomes for either antigen presentation or delivery. Post-isolation modification of exosomes can be performed through multiple methods including ultrasound incubation, freeze-thaw cycling, extrusion (Kooijmans *et al.*, 2016), and electroporation (Naseri *et al.*, 2018). Parental cell-based modification involves altering the exosome before extraction. Direct modification of exosome membrane protein genes can be applied to display specific targeting molecules (Liu *et al.*, 2023). For instance, a strategy called "exosome display" involves directing the antigen peptide of interest to the exosome membrane. This is achieved by introducing a transgene encoding the antigen, signal peptide, and exosome targeting domain (EDT) into the parental cell, resulting in the expression of the protein on the exosome surface during its biogenesis (Delcayre *et al.*, 2005). As a prove for the efficiency of this strategy, a plasmid construct containing enhanced green fluorescent protein (EGFP) fused to the C1C2 domain -a widely used EDT- is used to transfect in-vitro

Table 2: Advantages and limitations of potential novel technologies for vaccine design.

Technology	Benefits	Limitations
Exosome-based Vaccines	- Targeted Delivery: Exosomes naturally target immune cells, which enhances the delivery and effectiveness of the vaccine.	- Scalability Issues: Producing exosomes in large quantities with consistent quality remains a significant challenge.
	- Nano-Adjuvant Potential: Exosomes can function as nano-adjuvants, boosting the immune response without needing additional adjuvants.	- Antigen Loading Consistency: Achieving a consistent and adequate loading of antigens within exosomes is difficult, which may affect vaccine efficacy.
	- Versatile Cargo Loading: They can encapsulate proteins, lipids, RNA, and DNA, providing flexibility in vaccine design.	- Ethical and Safety Concerns: The introduction of exosomes into the body may interfere with natural cellular communication and pose unforeseen risks.
	- Surface Modification: Exosomes can be engineered to display specific antigens on their surface, improving targeting and immune response.	- Short Half-Life: Naturally derived exosomes often have a short half-life in circulation, requiring modifications to extend their stability and effectiveness.
Cell-Free Protein Synthesis (CFPS)	- Rapid Protein Production: CFPS can produce proteins within hours, accelerating the development and testing of vaccine candidates.	- Lower Yield: CFPS systems often produce lower yields of protein compared to traditional cell-based methods, which can limit their use in large-scale production.
	- Safety: The absence of live cells or pathogens in CFPS reduces the risk of contamination with virulent material, enhancing safety.	- Cost and Complexity: While CFPS can be scaled, the cost and complexity increase significantly, particularly when producing complex eukaryotic proteins or large quantities.
	- Scalability and Flexibility: CFPS systems can be easily scaled and adapted to produce different proteins, making them versatile for various vaccine types.	- Endotoxin Contamination: Bacterial CFPS systems can lead to endotoxin contamination, which is a significant concern for vaccine safety and requires additional processing steps.
	- Open System: The open nature of CFPS allows easy modification of the protein synthesis environment, enabling the incorporation of non-canonical amino acids or other modifications to enhance vaccine properties.	- Limited Post-Translational Modifications: CFPS systems, especially prokaryotic ones, may lack the machinery needed for complex post-translational modifications, which are essential for some vaccine proteins.
Self-Amplifying RNA (saRNA) Vaccines	- Dose Efficiency: saRNA vaccines require lower initial doses because the RNA amplifies within the host cells, reducing the amount of vaccine needed per dose.	- Safety Concerns: The replicative nature of saRNA raises concerns, particularly in immunocompromised individuals or pregnant women, where the vaccine might persist longer than desired.
	- Enhanced Immune Response: The self-amplification process generates both the antigen and immune-stimulating RNA forms, leading to a strong and prolonged immune response.	- Excessive Immune Response: The continuous amplification of RNA can sometimes lead to an excessive immune response, potentially counteracting the vaccine's benefits or causing adverse effects.
	- Rapid Development: Like other RNA vaccines, saRNA can be quickly designed and produced, which is crucial during outbreaks or pandemics.	- Stability Issues: The large size and complex structure of saRNA make it more prone to degradation, requiring sophisticated delivery systems to maintain its integrity.
	- Potential for Multivalency: saRNA can be engineered to include multiple antigens, potentially offering protection against various strains or pathogens in a single vaccine.	- Manufacturing Complexity: The production and delivery of saRNA are more complex than non-replicating RNA vaccines, increasing the cost and development time.
Artificial Intelligence (AI) and Computational Biology	- Accelerated Vaccine Design: AI can analyze large datasets to predict optimal antigenic sequences and design effective vaccines faster than traditional methods.	- Data Dependency: The effectiveness of AI in vaccine design is limited by the quality and quantity of available data. Incomplete or biased datasets can lead to inaccurate predictions.
	- Antigenic Variation Prediction: AI models can predict how pathogens might mutate, allowing for the design of vaccines that remain effective against future strains.	- Overfitting Risks: AI models can sometimes become too tailored to specific datasets, reducing their generalizability and predictive power in real-world applications.
	- Cost Reduction: By reducing the need for extensive lab work, AI can lower the overall cost of vaccine development.	- High Resource Requirement: Developing and running AI models requires significant computational resources, which may not be accessible to all research facilities.

- Enhanced Protein Structure Prediction: Computational tools can accurately predict protein structures, aiding in the design of vaccines that elicit strong immune responses.

- Limited Experimental Validation: While AI predictions can be powerful, they still require extensive experimental validation to ensure accuracy, which can be time-consuming and costly.

cultured cells. Exosomes extracted from those cells have shown the display of EGFP at the exosome surface. Exosomes generated from transfected cells with an adenovirus expression vector containing the EGFP-C1C2 transgene showed a great enhancement for humoral immune response in mice against EGFP when administrated intranasally and intramuscularly (Bliss *et al.*, 2020).

Another approach used the tetraspanins – a conserved family of transmembrane proteins that play essential functions such as protein trafficking (Susa *et al.*, 2024) – to address antigens to exosomes. It is suitable for displaying antigens bound to their extravesicular or intravesicular domains of exosomal surface (Stickney *et al.*, 2016). This method showed alterations in surface protein profiles and changes in the physicochemical properties of collected exosomes (J. Zhang *et al.*, 2022).

Transmembrane proteins that aid in the antigen display on exosome protein for antigen presentation are brain-abundant membrane-attached signal protein-1 (BASP1), lysosome-associated membrane protein-2b (LAMP-2B), and prostaglandin f2 receptor negative regulator (PTGFRN) (Dooley *et al.*, 2021; Xu *et al.*, 2021). Besides the role of LAMP-2B in displaying antigens on the surface of exosomes, it has also been used to test the capabilities of exosomes for specific cellular targeting. LAMP-2B-fused with rabies glycoprotein peptide was able to traffic the loaded exosomes toward neurocytes (Alvarez-Erviti *et al.*, 2011; El Safadi *et al.*, 2024).

Chemical modification is another approach for directly engineering the exosome allowing it to load various types of molecules. This method depends on adding and chemically modifying the exosome membrane's lipids and proteins. Those membrane molecules are modified by chemical ligation, affinity binding, lipid insertion, and enzymatic conjugation. The modified molecules interact with target peptides to be displayed on the exosome membrane (Malekian *et al.*, 2023). The chemical modification approach was used as a strategy termed the "cloaking" strategy for antigen displaying on exosome surface. It uses phospholipid membrane anchors and streptavidin to coat the surface of the exosomes, enabling the binding of biotinylated antigens and inducing antigen presentation as well as enabling targeted delivery to host cells and tissues (Antes *et al.*, 2018).

Additionally, molecules of interest can be loaded to the exosome lumen using molecular sorting modules (MSMs), a specific network of cellular proteins that help in delivering

molecules to the cellular compartment, which bind to molecules of interest and escort them to the exosome's lumen (Jafari *et al.*, 2020).

The implementation of these strategies has facilitated the advancement and enhancement of effective vaccinations for diverse viral diseases through the utilization of exosome technology. For instance, the Hepatitis C virus (HCV) non-structural 3 (NS3) protein-loaded exosome showed the ability to induce the production of specific memory CD8+ T-cells (Anticoli *et al.*, 2016). Furthermore, exosomes loaded with antigens from viruses such as influenza, Ebola, Crimean Congo hemorrhagic fever, and West Nile fever proved to provoke antigen-specific CD8+ T-cell response (Jungbauer, 2018). mRNA encodes for SARS-CoV-2 receptor-binding domain (RBD) were also loaded onto bovine milk-derived exosomes or lung-derived exosomes showed stimulated production of specific antibodies IgG and IgA (Popowski *et al.*, 2022; Q. Zhang *et al.*, 2023).

Exosome technology embraces many advantages making them a good candidate for advancing vaccine production, especially for challenging viruses like FMDV. Recent research has successfully utilized dendritic cell-derived EVs that have been stimulated by an inactivated FMD vaccine. The EVs contained epitopes that stimulated both B and T cells, resulting in a specific B cell response against FMDV and an indirect effect on T cells. (Menay *et al.*, 2024).

Exosomes still face multiple challenges that need to be addressed. These challenges include formulation issues such as producing exosomes with a consistent and adequate number of antigens (Schorey *et al.*, 2015), the requirement of a high throughput cellular system for large-scale production of exosomes, and the difficulty of controlling the diverse content of cargo unlike liposomes (Schwab *et al.*, 2015). Moreover, the introduction of exosomes to the host body may create a competitive situation with the exosomes produced by the host, thereby impacting the communication network or the therapeutic efficacy of the injected exosomes (Marcus and Leonard, 2013). Furthermore, ethical concerns were raised around the exosome regarding their safety and unpredictable consequences.

The unique characteristics of exosome technology such as their biocompatibility and their unique specificity toward immune cells, give a great potential for advancing the adjuvant and delivery system of FMD vaccines. However, the challenges related to scalability and control of ex-

osome content should be carefully studied and addressed to optimize exosome efficacy. With continuous research on exosome technology, we can harness their power for effective vaccine production and development (El Safadi *et al.*, 2024).

CELL-FREE PROTEIN SYNTHESIS (CFPS) SYSTEMS FOR VACCINE DEVELOPMENT

CFPS systems offer a promising platform to overcome the challenges of current vaccine production strategies. CFPS is a crude extract of cellular components that contains the machinery for transcription and translation allowing for protein synthesis in vitro with no need for intact host cells (Hu and Kamat, 2023). CFPS has been employed in various biological applications including but not limited to biosensing (Zhang *et al.*, 2020), high-throughput prototyping (Silverman *et al.*, 2020), and therapeutic and metabolic engineering (Chiba *et al.*, 2021; Ji *et al.*, 2022). However, vaccine development is an emerging area where CFPS shows great potential.

The biochemical characteristics of CFPS have made it an intuitive platform for vaccine development. CFPS provides a highly controllable environment where its resources are made available solely for protein production (Ranji *et al.*, 2013). Those resources can be modified through the incorporation of non-canonical amino acids (ncAAs) into the synthesized protein to increase its functionality (Ranji Charna *et al.*, 2022). The open nature of the CFPS systems allows for the rapid introduction of recombinant DNA reducing the time scale required for the rapid production of protein from days to hours (Liu *et al.*, 2019; Rosa *et al.*, 2021). The presence of no living pathogen or host for protein synthesis in the CFPS systems makes it safer than other methods and removes the risk of virulent material contamination (Fuenmayor *et al.*, 2017). Furthermore, CFPS components could be lyophilized through freeze-drying of CFPS facilitating the movement of the platform with no need for a specific cold chain setup (Stark *et al.*, 2021).

There are various types of CFPS extracts based on the required application. Prokaryotic cells, such as *E. coli*, are often used in CFPS production due to their easy production, cost-effectiveness, and scalability (Batista *et al.*, 2021). However, produced transmembrane proteins are prone to aggregation if not supplemented with a suitable solubilizing agent, and there is a potential for synthesized product contamination with endotoxins (Dondapati *et al.*, 2020). Eukaryotic-based CFPS is better at producing complex proteins than other bacterial CFPS as they contain endosomes that allow post-translation modifications. Yet, the Eukaryotic-based CFPS preparation procedure is complex, produces lower protein yield, and is more expensive than bacterial-based CFPS (Batista *et al.*, 2021; Dondapati

et al., 2020).

Various studies have been applied to address the challenges associated with the CFPS systems. For instance, the presence of lipopolysaccharides (LPS) and endotoxins can cause septic shock, this can be eliminated by using Triton X-114 for the extraction of endotoxins. Also, genetically modified bacterial strains such as ClearColi BL21 DE3 could be used where LPS structures were modified to reduce toxicity (Wilding *et al.*, 2019a). Moreover, to overcome post-translation modification limitation associated with the bacterial-based CFPS systems, protein disulfide isomerase or disulfide bond C (DsbC) could be added to aid the formation of disulfide bonds. Some *E. coli*-engineered cells like SHuffle, have oxidizing environments and contain cytoplasmic DsbC to support disulfide bond formation (Cole *et al.*, 2020; Dondapati *et al.*, 2020; Wilding *et al.*, 2019a). Membrane-bound glycosylation machinery such as PglB- or PglO-enriched extracts were also reported to improve glycoprotein titers (Hershowe *et al.*, 2021). Furthermore, lyoprotectants could be added to improve freeze-dried CFPS for therapeutic and conjugate vaccine production (Warfel *et al.*, 2023; Wilding *et al.*, 2019b).

CFPS has been employed in the development of different types of vaccines, including the subunit vaccine of influenza virus hemagglutinin (Lu *et al.*, 2014), heavy chain protein of botulinum toxins (Zichel *et al.*, 2010), the coat protein of the nervous necrosis virus (Kim *et al.*, 2015), and invasion plasmid antigens A, B, and H of *Shigella* spp. (Kappoor *et al.*, 2022). These vaccines were synthesized using *E. coli*-based CFPS systems. Other lysates were also used for the development of subunit vaccines including wheat germ lysates to develop *Plasmodium falciparum* vaccine using fragments from pfs230 (Tachibana *et al.*, 2011). Likewise, rabbit reticulocyte lysate was used to develop *Fasciola hepatica* vaccine using the SAP2 fragment (Ramos-Benítez *et al.*, 2018). CFPS was also used to enhance subunit vaccine efficacy by conjugating adjuvants directly to the antigen (Weiss *et al.*, 2021).

On-demand conjugate vaccines were synthesized using the in-vitro conjugate vaccine expression (iVAX) platform (Stark *et al.*, 2021). CFPS systems have also been employed to enhance the yield of VLP proteins. For Example, the Expression of bacteriophage MB2 coat protein using the CFPS system produced VLPs 14 times more than cellular systems (Bundy *et al.*, 2008; Patel and Swartz, 2011). Human norovirus VLP was created by using two capsid proteins in an *E. coli*-based CFPS system (Sheng *et al.*, 2017). CFPS was also used as a screening tool to find the most optimized constructs for best VLP assembly and yield. This method was used to explore various constructs for optimized HBV antigen-based VLPs (Colant *et al.*, 2021). Although of challenges associated with CFPS systems,

they hold various advantages over traditional vaccine production methods. CFPS systems tolerate the rapid screening and prototyping of vaccine candidates, as the protein expression required period is much shorter than cell-based systems. CFPS systems also have a more controlled environment for protein synthesis, which allows the production of difficult-to-express proteins in live cells. However, some factors should be considered such as efficiency, cost, and scalability when comparing CFPS systems to traditional cell-based methods. There are some limitations faced by CFPS systems regarding the cost of production, especially at larger production scales. Traditional methods, on the other hand, have been optimized over decades and can have higher protein yield however they require longer development time and complex infrastructure (Hu and Kamat, 2023).

In conclusion, CFPS platforms have the potential to completely transform the vaccine production system for the FMDV. The unique openness of the CFPS system provides a valuable means for making adaptable modifications to FMDV vaccinations, particularly during instances of outbreaks and epidemics. Research has been able to address some of CFPS systems challenges, such as efforts done to overcome endotoxin contamination and post-translation modification. With continuous investigation and development of CFPS systems, it would make them a viable alternative to traditional methods and can be integrated into the vaccine development pipeline.

SELF-AMPLIFYING RNA (saRNA) VACCINE

saRNA vaccine is an enhanced form of mRNA vaccine that includes the mRNA sequence in addition to a sequence that encodes four non-structure proteins (nsPs 1-4). These proteins enable the amplification of RNA copies within the cellular cytosol leading to the increase in the expressed antigen level. Hence, this method requires a lower initial dose of vaccine than non-replicating mRNA that can induce the same level of immune response (Lokras *et al.*, 2024). In addition to the formation of antigen encoded by mRNA sequence, The intracellular amplification process provokes the immune response due to the generation of different types of nucleic acids such as single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), which act as pathogen-associated molecular patterns (PAMPs). This leads to a pathogen-specific immune response, consisting of a high level of type 1 interferons (IFNs) (Geall *et al.*, 2012; McNab *et al.*, 2015).

The nsPs in saRNA vaccine encode for the replicase enzyme of the Venezuelan equine encephalitis virus (VEEV). This construct also includes different untranslated elements such as 5' UTR, 3' UTR, and poly-A tail and two coding sequences that are expressed into the antigen-of-interest and RNA-dependent RNA polymerase (RDRP). The RDRP

enzyme is used to synthesize a complementary negative RNA strand to the positive saRNA strand which will then serve as a template to generate more RNA-positive strands and the cycle goes on (Lokras *et al.*, 2024; Maruggi *et al.*, 2019). A recent saRNA vaccine against SARS-CoV-2 was approved in Japan for human use (Oda *et al.*, 2024). An influenza vaccine based on saRNA has been in development and showed an adequate level of antigen production with lower RNA doses (Cheung *et al.*, 2023). These examples highlight the potential use of saRNA technology in the production of effective vaccines with reduced dosages.

saRNA vaccines face several drawbacks that have led to the termination of vaccine development against different pathogens. The main challenge faced the self-amplifying RNA, is concern regard the replicative nature of the vaccine. The concern is raised due to the possible adverse effects on vulnerable individuals such as immunocompromised individuals and pregnant women. In immunocompromised patients, the vaccine may persist as its clearance is less efficient. Concern related to pregnant women is the use of genetic material derived from viruses that cause congenital infections. However, there is a low chance of transferring the RNA vaccine to the fetus, as the route of administration is usually intramuscular or intravenous injection and distribution will be hindered by the placenta (Comes *et al.*, 2023). Another challenge resembling the continuous amplification of RNA strands could lead to excessive IFN response, that counteract the saRNA amplification and expression (Pepini *et al.*, 2017). In addition, the large strand size of saRNA provides a more complex secondary structure and affects the cost and development of saRNA-based vaccines. Its large size also increases the risk of strand hydrolysis and lipid RNA formation which could either inactivate the saRNA or reduce its efficacy (Lokras *et al.*, 2024; Packer *et al.*, 2021). Finally, recent data have shown that some nucleotide analogs can be used to modify the saRNA to improve its performance and decrease the adverse effects of saRNA, which was not possible before and hindered its development (Lokras *et al.*, 2024; McGee *et al.*, 2024).

A different derivative of saRNA is the trans-amplifying RNA (taRNA). In this method, two ORFs are present on separate strands where the antigen of interest is amplified from one strand using the replicase enzyme expressed from the other strand. This approach provides specific amplification due to the presence of a specific sequence element that can only be recognized by the replicase enzyme (Lundstrom, 2023) Figure 3. taRNA has many advantages over saRNA making it a promising platform that can be used for the development of the FMDV vaccine. One of the advantages of the taRNA is the ability to combine multiple antigen sequences to produce multivalent vaccines since every strand can be synthesized independently giving a chance for continuous update of the vaccine with new anti-

gens representing circulating strains. Moreover, nucleotide modification is also possible which can reduce innate immune response and enhance protein expression (Schmidt *et al.*, 2022). In addition, taRNA showed a reduced risk of developing excessive immune response, as replication machinery separated from the antigen so no overexpression is expected. Still, the use of multiple RNA strands may impose a challenge in developing an effective delivery method of the vaccine. Those advantages provide insights for the development of FMD vaccines that can counteract the ever-changing outbreaks.

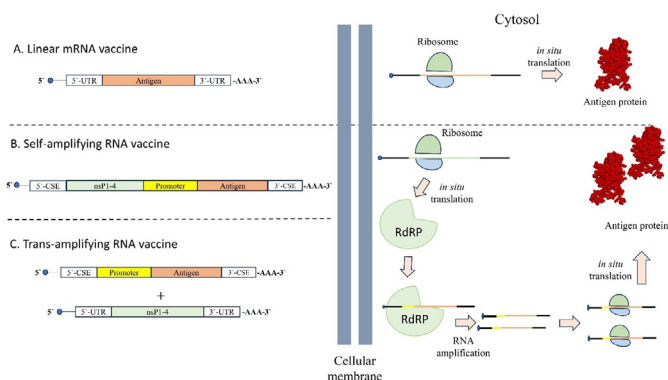


Figure 3: Different types of RNA vaccines; UTR: untranslated regions; CSE: conserved sequence element; nsP: non-structural protein; RdRP: RNA-dependent RNA Polymerase.

In summary, self-amplifying RNA vaccines are still an evolving field that suffers from some drawback such as excessive immune responses and instability that need to be addressed to ensure their safety and feasibility. the comparison between saRNA and taRNA showed the need for continuation of research for optimization of their design and implementation. The amplifying RNA provide insights for the development of FMD vaccines that can counteract the ever-changing outbreaks.

USE OF ARTIFICIAL INTELLIGENCE (AI) AND COMPUTATIONAL BIOLOGY

The application of computational biology and AI addresses some major concerns of vaccine development. For instance, these advanced approaches were employed for the development of multiepitope vaccines for various pathogens such as SARS-CoV-2 (Bhattacharya *et al.*, 2023). The process mainly involves the usage of genomic and immunology information that has been previously identified and present on databases or web servers. These datasets were also used to train machine learning (ML) or deep learning (DL) models for the prediction of antigenic sequences or segments that have not been previously identified. A variety of tools, databases, and web servers are listed in Table 3.

Computational vaccine development usually focuses on RNA, DNA, VLP, peptide, and subunit-based vaccines. These vaccines often depend on the use of structural proteins of the target pathogen in their formulation (Ananya *et al.*, 2024; Massignani *et al.*, 2019; Sesterhenn *et al.*, 2020). After the selection of the proper protein sequences, a pipeline is developed for predicting epitopes that have a binding affinity towards receptors present on immune cells. This prediction is based on either amino acid physicochemical properties or propensity scale. Correct selection of epitopes can significantly aid the reduction of required time to identify correct vaccinal strains (Kogay and Schönbach, 2019). Moreover, predicting protein structure, mainly tertiary structure, is crucial for understanding of conformational properties of the protein. Various computational tools can predict tertiary structure through either homology modeling or de novo prediction. The predicted tertiary structures could be then optimized and validated for their amino acid position (Kelley *et al.*, 2015; Zipkin, 2021). They can also be validated for their interaction and stability through molecular docking and simulation (Agu *et al.*, 2023; Vidal-Limon *et al.*, 2022).

ML and DL models trained on antigenic variation data and vaccine-matching results of FMDV isolates could be employed to identify and predict the most suitable vaccine for the circulating strains. However, these models are limited by the availability of protein sequences or experimental vaccine-matching results even with high model accuracy (Qiu *et al.*, 2021).

Several limitations may impose some challenges in the development of ML and DL models for vaccine development (Bravi, 2024). A major limitation resembles on development of an effective ML model uses high-quality annotated datasets. The population of available immunology data has extreme diversity, ranging from epitope cross-reactivity, conformational diversity, and multiple binding modes, which adds a great challenge for representative sampling for model training (Bradley and Thomas, 2019). Moreover, the available data may lack important information for model training such as sequence and structural data (Bravi, 2024). Some datasets may have an over-representation of some epitopes due to their biomedical interest which might enforce sampling bias on training datasets (Hudson *et al.*, 2023). The limitation of such data can hinder the training of correct models and may affect the accuracy of model predictions.

Another limitation may present in the overfitting of the trained models. This limitation can affect the model generalization and predictive power in real-world applications (Gygi *et al.*, 2023). Model validation is important to ensure the generalization of the model with no overfitting problems. Model validation involves testing on independent

Table 3: List of Computational biology and AI web servers and software used for vaccine development.

Domain	Name	Description	Reference
Immunological and structure databases and webservers.			
Immunological databases	AntiJen	The database contains experimentally obtained quantitative binding data for peptides binding to MHC ligands, TCR-MHC complexes, T and B-cell epitopes, and other related information.	(Toseland <i>et al.</i> , 2005)
	AntigenDB	The antigen selection database has data on over 400 protein antigens, four glycoprotein antigens, and 13 lipoprotein antigens.	(Ansari <i>et al.</i> , 2010)
	IMGT	This database provides information on immunoglobulins (Ig) or antibodies, T-cell receptors (TCR), major histocompatibility complex (MHC) in human and other vertebrate species, as well as proteins of the immunoglobulin superfamily (IgSF), MH superfamily (MhSF), and other related proteins of the immune system.	(Lefranc, 2001)
	MUGEN	A mouse model database of human immunological diseases	(Aidinis <i>et al.</i> , 2007)
	Mosaic Vaccine Designer	Vaccine candidate protein sequence design access and design	(Thurmond <i>et al.</i> , 2008)
Membrane protein	HMMpTM	Using the Hidden Markov Model method, it predicts the structural arrangement of transmembrane proteins while detecting specific phosphorylation and glycosylation sites	(Tsaousis <i>et al.</i> , 2014)
	DeepTM-HMM	It is a web-based tool that combines deep learning algorithms with Hidden Markov Models to predict transmembrane helices in protein sequences accurately	(Gao <i>et al.</i> , 2023)
	PDBTM	A subset of PDB for transmembrane proteins that scans PDB entries using the TMDEt algorithm	(Kozma <i>et al.</i> , 2012)
	Mpstruc	Structural database for peer-reviewed protein structures	(Shimizu <i>et al.</i> , 2018)
	Membranome	Provides structural and functional data on bitopic transmembrane proteins	(Lomize <i>et al.</i> , 2017)
Surface protein	SurfaceGenie	Integrates a consensus-based prediction of cell surface localization with user-input data to prioritise candidate cell-type specific surface markers	(Waas <i>et al.</i> , 2020)
	Surface ID	Geometric deep learning approach for fast surface comparison using geometric and chemical features	(Riahi <i>et al.</i> , 2023)
	Cell Surface Protein Atlas	Database of cellular surface proteins	(Bausch-Fluck <i>et al.</i> , 2015)
Epitope prediction			
continuous B-cell epitopes	Bcepred	The BcePred server utilizes the physico-chemical characteristics of amino acids to anticipate B cell epitopes. The qualities that are commonly associated with B cell epitopes are hydrophilicity, flexibility, accessibility, polarity, exposed surface, and twists. The quantification of these qualities is established by assigning a numerical value to each of the 20 naturally occurring amino acids. The server demonstrates a predictive capability of identifying epitopes with an accuracy of 58.7% by employing a combination of approaches, while maintaining a threshold of 2.38.	(Saha and Raghava, 2004)
	BepiPred	BepiPred-2.0 utilizes a random forest method that has been trained on epitopes derived from antibody-antigen protein structures. The method demonstrated superior performance compared to other existing tools in predicting epitopes based on both solved 3D structures and a huge dataset of linear epitopes obtained from the IEDB database.	(Jespersen <i>et al.</i> , 2017)

	ABCPred	The purpose of the ABCpred service is to utilize an artificial neural network to predict B cell epitope(s) in a given antigen sequence. The dataset utilized for both training and testing comprises 700 B-cell epitopes and 700 non B-cell epitopes, which are random peptides with a maximum length of 20 residues. After experimenting with several neural networks, we were able to achieve an accuracy of 65.93% by utilizing a recurrent neural network.	(Saha and Raghava, 2006)
Prediction of discontinuous B-cell epitopes	EPCEs	Antigen Epitope Prediction approach utilizes six distinct scoring functions: residue epitope propensity, conservation score, side-chain energy score, contact number, surface planarity score, and secondary structure composition.	(Liang <i>et al.</i> , 2020)
	DiscoTope	The DiscoTope service utilizes protein three-dimensional structures to predict B-cell epitopes. DiscoTope version 3.0 employs enhanced B-cell epitope prediction by utilizing AlphaFold2 modeling and inverse folding latent representations.	(Haste Andersen <i>et al.</i> , 2006)
T-cell epitopes	EpiJen	Predict T cell epitopes using multiple steps. The technique is employed on a collection of overlapping peptides derived from a whole protein sequence and functions as a sequence of filters that effectively decrease the quantity of possible epitopes.	(Bhasin and Raghava, 2007)
	NetMHC-3.0	Trained model utilizing a substantial amount of quantitative peptide data, which included both affinity data from the Immune Epitope Database and Analysis Resource (IEDB) and elution data from SYFPEITHI. The model produces highly accurate predictions of the interaction between major histocompatibility complex (MHC) and peptides. Artificial neural networks (ANNs) have undergone training for a total of 81 distinct human major histocompatibility complex (MHC) alleles, which include HLA-A, HLA-B, HLA-C, and HLA-E. Moreover, forecasts for 41 animal alleles (Monkey, Cattle, Pig, and Mouse)	(Andreatta and Nielsen, 2016)
	MHC2Pred	A Support Vector Machine (SVM) approach is used to forecast peptides that have a high likelihood of binding to several Major Histocompatibility Complex (MHC) class II molecules. The SVM-based technique achieves an average accuracy of approximately 80% for 42 alleles. The method's performance was inferior for a few alleles due to the smaller amount of the dataset. The efficacy of the approach was evaluated using 5-fold cross-validation.	(Lata <i>et al.</i> , 2007)
	SVMHC	The SVMHC server is capable of predicting peptides that bind to both MHC class I and class II molecules. The system provides rapid analysis of a diverse array of genetic variations, and presents prediction outcomes in multiple comprehensive formats. The server can be utilized to identify the most probable molecules that attach to a protein sequence and to examine the impacts of single nucleotide polymorphisms in relation to MHC-peptide attachment.	(Donnes and Kohlbacher, 2006)
Protein Tertiary structure and visualization			
Homology modeling	HHpred	The server is designed to do distant protein homology recognition and structure prediction quickly. It is also the first to provide pairwise comparison of profile hidden Markov models (HMMs).	(Gabler <i>et al.</i> , 2020)
	MODELLER	Utilized for the homology or comparative modeling of protein tertiary structures.	(Eswar <i>et al.</i> , 2006)
De-novo/ab initio modeling	Raptor-X	This method allows for the prediction of the tertiary structure and contact information of protein sequences that do not have closely related homologs in the Protein Data Bank (PDB). RaptorX utilizes advanced algorithms to forecast the secondary and tertiary structures of proteins, as well as anticipate contact and distance maps, solvent accessibility, disordered regions, functional annotation, and binding sites.	(J. Xu <i>et al.</i> , 2021)
	I-TASSER	Protein structure prediction and structure-based function annotation are accomplished using a hierarchical method.	(Zhou <i>et al.</i> , 2022)

	ROSSETA	The Monte Carlo approach is used to assemble small segments of known proteins, resulting in the production of protein conformations that closely resemble their natural structure.	(RosettaCommons. n.d, 2024)
	Alpha Fold	Google DeepMind has created an AI system that can predict the three-dimensional structure of a protein based on its sequence of amino acids. It consistently achieves accuracy that is comparable to experimental results.	(Varadi <i>et al.</i> , 2022)
	ModLoop	Modeling of Loops in Protein Structures Protein	(Fiser and Sali, 2003)
Structure visualization	Pymol	This is a robust and all-encompassing software tool that is used for creating realistic and dynamic 3D representations of molecular structures.	(Yuan <i>et al.</i> , 2017)
	VMD	A software application designed for the visualization, animation, and analysis of complex biomolecular systems using three-dimensional graphics and integrated scripting capabilities.	(Spivak <i>et al.</i> , 2023)
	Chimera	platform allows for the dynamic representation and examination of molecular structures and associated data, such as density maps, trajectories, and sequence alignments.	(Meng <i>et al.</i> , 2006)
Molecular docking and dynamic simulation			
Molecular docking	Autodock	Optimisation of ligands within a receptor binding site using Lamarckian genetic algorithm	(Rizvi <i>et al.</i> , 2013)
	MolDock	Evaluate ligands and receptor binding affinity using the Fast Fourier transform (FFT) algorithm, Input: PDB, MOL2, and SDF It is unsuitable for multi-site binding pockets and is less accurate in predicting binding modes and interactions, affecting the reliability of docking outcomes	(De Azevedo Jr., 2010)
	CLUSPro	Web server and stand-alone versions are available for use in specialized antibody mode	(Kozakov <i>et al.</i> , 2017)
	HADDOCK	High Ambiguity Driven DOCKing is a powerful software for protein-protein docking that uses experimental data to allow the flexibility of small receptor changes due to the ligands' interaction	(Saponaro <i>et al.</i> , 2020)
	PatchDock	Geometric hashing algorithms are used to predict protein-protein interaction. Users are offered to select Antibody-antigen	(Schneidman-Duhovny <i>et al.</i> , 2005)
Molecular dynamic simulation	GROMACS	The program is a versatile tool for conducting molecular dynamics simulations, specifically for simulating the Newtonian equations of motion in systems containing hundreds to millions of particles. It is a project that is driven by the community.	(Abraham <i>et al.</i> , 2015)
	Amber	A suite of software applications that execute biomolecular simulations at the atomic scale, predominantly utilizing molecular dynamics.	(Case <i>et al.</i> , 2023)
	TINKER	A comprehensive and versatile software suite for molecular mechanics and dynamics, specifically designed to cater to the unique requirements of biopolymers.	(Lagardère <i>et al.</i> , 2018)
	OpenMM	The simulation code stands out from others because to its exceptional combination of custom forces and integrators, openness, and remarkable performance, particularly on modern GPUs.	(Eastman <i>et al.</i> , 2017)

datasets and ensuring their strength to different input data. Still, a challenge is faced in obtaining such validation data. Additionally, AI models are resource-intensive and require a lot of computational resources that might not be available in most laboratories (Xu *et al.*, 2021). Finally, there are limited mentions of testing prediction results using experimental validation (Major *et al.*, 2020).

Computational biology and AI future in the vaccine industry is very promising in general and in FMDV vaccine development in particular. The quasispecies nature of FMDV necessitates the continuous development of its vaccines, which traditionally requires a lot of resources and time. The introduction of computational analysis in the cycle of FMDV vaccine production can shorten the required time,

lower costs, and increase vaccine efficacy, offering a significant advantage in fighting such a challenging virus. Besides, AI models can also develop to enhance and develop emerging technologies such as the assessment of exosome receptors for better categorization of its types, in-silico design and optimization of saRNA strands, and prediction of suitable antigens for production on CFPS systems.

CONCLUSIONS AND RECOMMENDATIONS

The necessity of utilizing innovative approaches capable of adapting to the rapid mutation and quasispecies existence of FMDV. This review highlights the complex nature of FMDV, including its different serotypes, structural characteristics, and the high mutation rate. Moreover, it discussed current vaccine strategies presenting their strong and weak points.

- Inactivated vaccines are a well-known and secure method but require several doses and are hindered by cross-protection problems among serotypes.
- Live-attenuated vaccines can induce strong immune responses but possess the risks of reversion to virulence requiring monitoring.
- VLPs mimic the virus structure without containing infectious material, enhancing safety while still promoting strong immune responses, but still no field data ensure its efficacy.
- Peptide-based vaccines offer flexibility in targeting certain immune responses but mostly need adjuvants to boost immunogenicity.
- RNA vaccines present a rapidly adaptable platform for designing vaccines through issues concerning stability and delivery.
- Emerging technologies such as exosome-based vaccines, cell-free protein synthesis systems, and the integration of artificial intelligence (AI) and computational biology into vaccine design further enhance the potential for innovative solutions to existing challenges. By leveraging these technologies, researchers can develop vaccines that not only provide broader protection against diverse FMDV strains but also improve stability, safety, and accessibility.

FUTURE DIRECTIONS

Looking ahead, several key areas of research and development are essential for advancing FMDV vaccine strategies: The development of universal vaccines should be the focal point of all efforts aimed at providing broad protection against several serotypes of FMDV. Hence it can be done by identifying conserved epitopes and employing advanced technologies like AI in predicting the antigenic variations. Socioeconomic barriers need to be addressed as per re-

search objectives for effective vaccine deployments in low- and middle-income countries. Therefore, cheap vaccines that do not entail such rigorous cold storage are necessary. For further advancements, there is a need to integrate AI with computational biology when designing and developing vaccines. Such advancements would encompass improved data collection and analysis for better model predictions as well as optimization of candidate vaccines.

By focusing on these future directions, researchers can enhance the effectiveness of FMDV vaccination strategies, ultimately contributing to the control and eradication of this economically significant disease in livestock populations worldwide. Through continued innovation and collaboration, the challenges posed by FMDV can be effectively addressed, leading to improved animal health and productivity.

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NOVELTY STATEMENT

This review highlights the point between traditional and emerging vaccine technologies for FMD. While the conventional vaccine approaches have been reviewed extensively, this review introduces cutting-edge advances like exosome-based vaccines, cell-free protein synthesis systems, self-amplifying RNA, and artificial intelligence vaccine design. The review discusses the innovative solutions technologies can provide to persistent challenges of FMDV such as viral mutation rate and antigenic variability.

AUTHOR'S CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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